



Isolation and characterization of an antifungal peptide from fruiting bodies of edible mushroom *Lentinus squarrosulus* Mont.

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ABSTRACT

Aims: To isolate and characterize an antimicrobial peptide from fruiting bodies of *Lentinus squarrosulus* Mont., the Thai common edible mushroom.

Methodology and results: Solid ammonium sulfate at 40-80% (w/v) final concentration was utilized to precipitate the proteins and further purified by ion exchange chromatography on DEAE-cellulose and gel filtration chromatography on Sephadex G-25. The peptide was adsorbed on DEAE-cellulose and Sephadex G-25. It appeared as a single band with a molecular mass of about 17 kDa (kilodalton) in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Further investigation of antimicrobial properties of purified peptide revealed that it has no activity against both Gram positive and Gram negative bacteria. However, it exhibited strong antifungal activity against various species of fungal pathogen of human. Among the high sensitive strains, *Trichophyton mentagrophytes*, *T. rubrum*, and *Candida tropicalis* are clinical isolates. Moreover, the potency was found to be concentration dependent and comparable with Ketoconazole, the commercial antifungal drug.

Conclusion, significance and impact study: In this work, the novel bioactive peptide from fruiting bodies of *L. squarrosulus* Mont. has been isolated. It shows potent activity against various clinical isolates of fungal pathogen of human. It may have potential for pharmaceutical application.

Keywords: Antifungal peptide, antifungal activity, *Lentinus squarrosulus* Mont., DEAE-cellulose, Sephadex G-25

INTRODUCTION

According to the emergence of antibiotic-resistant microbes which has become a major health threat over the past decades (Novak *et al.*, 1999; Spellberg *et al.*, 2008; Fischbach and Walsh, 2009), there is an urgent need to search for natural antibiotics. Therefore, interest in peptide antibiotics from natural source has increased greatly, as these are believed to be high potent, higher specificity, having less toxicity, do not accumulate in organs, and biological and chemical diverse (Glukhov *et al.*, 2005; Gordon and Romanowski, 2006; Lavery and Gilmore, 2014). Antimicrobial peptides represent a heterogeneous group of structurally diverse proteins produced by a wide range of organisms: mammals, insects, plants, fungi and bacteria (Bulet *et al.*, 1999; Zasloff, 2002).

Mushrooms, the macrofungi, have long been used as valuable food source and as traditional medicines around the world since ancient times (Guani-Guerra *et al.*, 2010). The proteins from these fungi have shown several biological activities including antifungal and antibacterial

effects (Ngai and Ng, 2003; Wang and Ng, 2004; 2006; Chu *et al.*, 2005; Wong *et al.*, 2011). Although there are numerous mushroom species, only a few have been studied for bioactive peptides. In Thailand, there are also various species of mushrooms including *Lentinus* spp. *Lentinus squarrosulus* Mont. or "Hed khon khao" in local language, is one of edible indigenous mushroom species and abundantly grown in North East of Thailand. The local Thai people used *L. squarrosulus* Mont. mushroom for food for long time (Royal Institute of Thailand, 1996). Although they were also used as curative or tonic agents in Thai traditional medicine, there were no study of bioactive peptide from this mushroom. However, there is a report of antifungal peptide from *Lentinus* spp. such as lentin, an antifungal peptide from *Lentinula edodes* (Berk.) Pegler (shiitake) (Ngai and Ng, 2003). The aim of this study was to isolate and characterize an antimicrobial peptide from *L. squarrosulus* Mont. which is the edible mushroom commonly found in Thailand.

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MATERIALS AND METHODS

Collection of mushroom sample

Lentinus squarrosulus Mont. were purchased from local markets in Bangkok, Thailand. They will be kept in sterile plastic bags and stored at $-20\text{ }^{\circ}\text{C}$ until isolation. They will be sent to the Department of Agriculture, Ministry of Agriculture and Cooperatives for phenotypic identification.

Extraction of crude protein from *L. squarrosulus* Mont.

Fresh mushrooms were cleaned with sterile distilled water and homogenized in distilled water (3 mL/g) using a blender (Guo *et al.*, 2005). After centrifugation of homogenate at 12,000 $\times g$, at $4\text{ }^{\circ}\text{C}$ for 30 min, the resulting supernatant was collected and added with Tris-HCl buffer (pH 7.4) until the concentration reached 10 mM. Then, solid ammonium sulfate was slowly added to reach about 0-40% and 40-80% saturation (Zhao *et al.*, 2009). After standing at $4\text{ }^{\circ}\text{C}$ for 1 h, the pellet was collected by centrifuge (12,000 $\times g$ for 30 min), then redissolved in 50 mL of 10 mM Tris-HCl buffer (pH 7.4). The solubilized protein pellet was dialyzed with 10 mM Tris-HCl buffer (pH 7.4) at $4\text{ }^{\circ}\text{C}$ overnight to remove ammonium sulfate. Crude proteins were obtained with freeze-drying method.

Isolation of antimicrobial peptide

Ion exchange chromatography on Diethylaminoethyl (DEAE) cellulose (Sigma-Aldrich) column (5 \times 15 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.4) was then carried out. An aliquot of 1 mL solubilized crude proteins obtained from ammonium sulfate precipitation step was layered onto the column and the proteins were allowed to bind with the matrix. After the unbound proteins had been washed with 10 mM Tris-HCl buffer (pH 7.4), the bound proteins were eluted by addition of a linear salt concentration gradient (0-1 M NaCl) in 10 mM Tris-HCl buffer (pH 7.4) at a flow rate of 0.2 mL/min. A total of 130 fractions (3 mL each) were collected and a small aliquot from each fraction was used to determine antimicrobial activity and the absorbance at 280 nm. Fractions with antimicrobial activity were pooled and concentrated using freeze-drying method. Further purification of pooled fractions with antimicrobial activity was carried out utilizing size exclusion chromatography on a Sephadex G-25 (Amersham Biosciences) column (5 \times 30 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.4). An aliquot of 500 μL of concentrated protein sample was applied into the column. The column was eluted with 10 mM Tris-HCl buffer (pH 7.4) at the same previously described flow rate. All purification steps were performed at $4\text{ }^{\circ}\text{C}$. Aliquot of each collected fraction was used to determine antimicrobial activity as well as the absorbance at 280 nm. Fractions with antimicrobial activity were pooled and concentrated using freeze-drying method. The concentrated proteins were further determined for

molecular weight, protein content and antimicrobial activity.

Determination of protein content

The protein contents in crude extract, solubilized ammonium sulphate precipitates and purified fractions of chromatography were estimated by Bradford method (Bradford, 1976).

Determination of molecular mass

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the homogeneity of purified fractions as well as estimate the molecular mass of purified peptide. The method was carried out in accordance with the method described by Laemmli and Favre, (1973) using a 14% (w/v) gel. The gels were stained with Coomassie brilliant blue R-250 and destained with methanol: acetic acid: water (30:10:60% v/v). The molecular mass of the purified peptide was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins (Invitrogen, USA).

Assay of antimicrobial activity

Test microorganisms

Yeast and mold (*Candida albicans* ATCC 10231 and *Aspergillus niger*) as well as Gram-positive (*Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633) and Gram negative bacteria (*Escherichia coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 4352 and *Pseudomonas aeruginosa* ATCC 15442) were obtained from Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Five filamentous fungi and yeast of clinical isolates (*Trichophyton mentagrophytes*, *T. rubrum*, *C. tropicalis*, *Microsporium gypseum* and *Epidermophyton floccosum*) were provided by the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Thailand. The fungal and bacterial cultures were propagated on Sabouraud Dextrose Agar (SDA) (Difco, USA) and Mueller Hinton Agar (MHA) (Difco, USA), respectively and activated by periodic sub-culturing at monthly intervals.

Assay for antimicrobial activity

The antimicrobial activity of solubilized ammonium sulphate precipitates and fractions of column chromatography was determined using the disc diffusion method (Collins and Lyne, 1987) with modification for both antibacterial and antifungal activity. Approximately, 30 mL of molten SDA or MHA were transferred to the sterile Petri dish and allowed to solidify. An aliquot of 100 μL of suspension containing approximately 10^6 CFU (colony forming units)/mL of bacteria, or 10^6 CFU/mL of yeasts or 10^6 conidia/mL of filamentous fungi (Nweze *et*

al., 2010) was spread uniformly over the dried agar surface with a sterile bent glass rod. After drying the plates at 37 °C for 1 h, sterile Whatman (UK) filter paper discs (6 mm in diameter) impregnated with 30 µL of sample solution containing various amount including 3.75, 7.5, 15, 30, 40, 50 µg of peptide per disc were placed on the surface of the inoculated agar. The antibiotic Amikacin (Oxoid, UK) 30 µg/disc and antifungal Ketoconazole (SIGMA, Singapore) 20 µg/disc were used as a positive control. Disc impregnated with only solvent (without protein) was used as negative control. These plates were incubated at 37 °C for 24 h for bacterial strains and at 30 °C for 24 and 72 h for yeast and filamentous fungi, respectively. The antimicrobial activity was evaluated by measuring the zone of inhibition in terms of millimeter. Each experiment was done in triplicate and the mean of clear zones was calculated.

RESULTS

Identification of mushroom *L. squarrosulus* Mont.

After collection of fresh mushrooms from local markets, they were packed in sterile plastic bag and sent to

Department of Agriculture, Ministry of Agriculture and Cooperatives for phenotypic identification. The identification results indicated that the samples belong to *L. squarrosulus* Mont.

Extraction of crude proteins

The crude proteins were precipitated from crude extract of *L. squarrosulus* Mont. fruiting bodies by slowly adding ammonium sulphate to final concentration of 0-40% (w/v) and 40-80% (w/v). The solubilized ammonium sulfate precipitates from 0-40% (w/v) saturation showed no antimicrobial activity, while solubilized precipitates from final concentration of 40-80% (w/v) exhibited antifungal activity against *C. albicans* ATCC 10231, *C. tropicalis*, *A. niger*, *T. mentagrophytes* and *T. rubrum* (Table 1). However, the antibacterial activity was not observed in both solubilized precipitates from 0-40% (w/v) saturation and 40-80% (w/v) saturation of ammonium sulphate (data not shown). The precipitation step with 40-80% (w/v) saturation of ammonium sulfate resulted in a recovery of about 64% (Table 2).

Table 1: Antifungal activity of samples from different stages of isolation and purification of *L. squarrosulus* Mont. fruiting bodies against various species of fungi.

Samples	Purification stages	Zone of inhibition (mm)*				
		<i>A. niger</i>	<i>T. mentagrophytes</i>	<i>T. rubrum</i>	<i>C. tropicalis</i>	<i>C. albicans</i>
Positive control (Ketoconazole)		14.83±0.29	24.81±0.36	24.3±0.16	22.34±0.81	23.15±0.28
Negative control (10 mM Tris-HCl pH 7.4)		0	0	0	0	0
Crude protein (40-80% (NH ₄) ₂ SO ₄ precipitate)	(NH ₄) ₂ SO ₄ precipitate	10.83±0.6	16.02±0.38	15.13±0.12	16.51±0.43	15.22±0.7
Positive control (Ketoconazole)		15.13±0.34	23.9±0.24	24.42±0.1	21.71±0.12	22.48±0.21
Negative control (10 mM Tris-HCl pH 7.4)		0	0	0	0	0
Eluted fraction 0.5 M NaCl (DEAE column)	DEAE column	11.81±0.92	18.11±0.65	17.28±0.47	18.62±0.5	18.39±0.63
Positive control (Ketoconazole)		14.17±0.8	25.84±0.27	25.88±0.51	22.8±0.21	22.37±0.35
Negative control (10 mM Tris-HCl pH 7.4)		0	0	0	0	0
Eluted fraction F4 (G-25 column)	G-25 column	12.64±0.5	25.7±0.36	22.81±0.43	20.54±0.35	20.62±0.82

Positive control (Ketoconazole), 20 µg/disc; Samples purification stage, 30 µg/disc and Negative control (10 mM Tris-HCl pH 7.4)
 * Means of clear zones ± SEM (n=3)

Table 2: Yield of protein with antifungal activity obtained from different stages of isolation and purification of *L. squarrosulus* Mont. fruiting bodies.

Stages of purification	Volume (mL)	Amount of proteins (mg) ^{a,b}	Recovery of protein (%)
Crude extract	480	331	100
Solubilized 40-80% of NH ₄ (SO ₄) ₂ precipitates	197	212	64.05
F3 (0.5 M NaCl eluted)	90	107	32.32
F4 (G-25 Sephadex column)	21	21	6.34

a, 1.5 kg fresh mushroom was used for purification.

b, Protein concentration determined by Bradford assay using BSA as a standard protein.

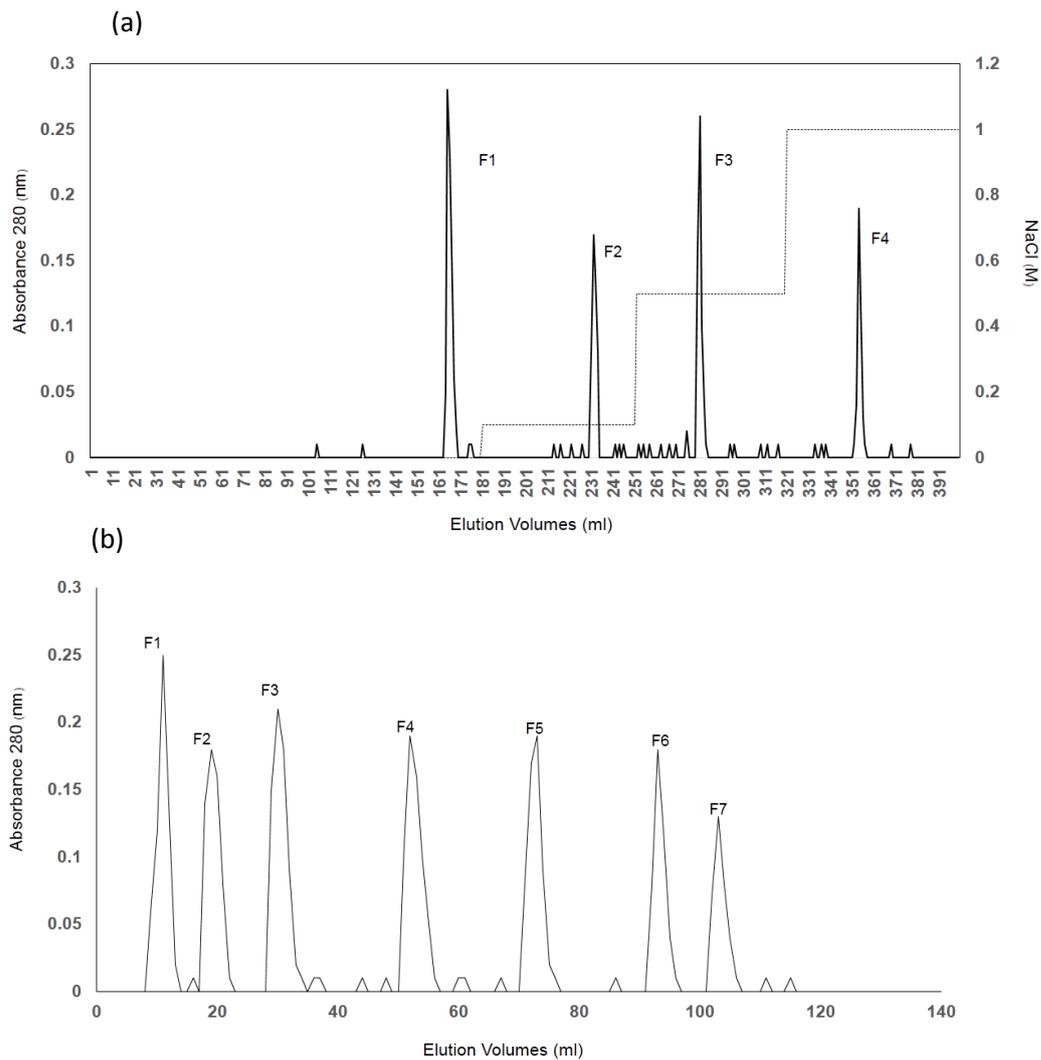


Figure 1: The column chromatographic purification of solubilized ammonium sulphate precipitates from crude extract of *L. squarrosulus* Mont. fruiting bodies. a, Ion exchange chromatography of solubilized ammonium sulphate precipitates from crude extract of *L. squarrosulus* Mont. fruiting bodies on a DEAE-cellulose column (5 × 15 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4) at a flow rate of 0.2 mL/min. The adsorbed proteins were eluted sequentially with 0.1 to 1.0 M NaCl in the same buffer to yield fractions F2, F3 and F4. Only fraction F3 (eluted with 0.5 M NaCl) showed antifungal activity and b, Gel filtration of fraction F3 (from DEAE-cellulose column) on a Sephadex G-25 column (5 × 30 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4) at a flow rate of 0.2 mL/min. The peak labeled F4 exhibited antifungal activity.

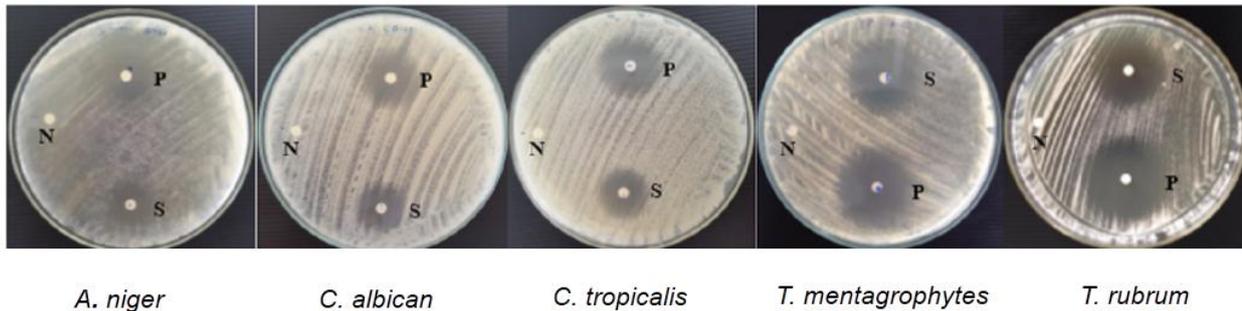


Figure 2: Antifungal activity of purified peptide toward *A. niger*, *C. albicans*, *C. tropicalis*, *T. mentagrophytes* and *T. rubrum*. P, Ketoconazole was used as positive control (20 µg); N, 10 mM Tris-HCl buffer (pH 7.4) used as negative control and S, eluted sample peptide from G-25 column. Each sample was tested with 30 µg purified peptide in 10 mM Tris-HCl buffer (pH 7.4).

Isolation of antifungal peptide

The solubilized crude protein from precipitation step with 40-80% (w/v) saturation of ammonium sulfate was further purified by ion-exchange chromatography on a DEAE-cellulose column with a stepwise salt gradient elution from 0.1 to 1.0 M NaCl. The elution profile obtained from DEAE-cellulose using a linear gradient of 0.1-1.0 M NaCl in 10 mM Tris-HCl buffer, pH 7.4 is shown in Figure 1a. Three main adsorbed peaks designated as F2, F3 and F4 (eluted with 0.1 M, 0.5 M and 1.0 M NaCl in 10 mM Tris-HCl buffer, pH 7.4) and one unadsorbed peak (F1) were obtained. All fractions were checked separately for activity. The antifungal activity was detectable only in adsorbed fractions eluted with 0.5 M NaCl, which correspond to peak F3. The purified protein exhibited strong antifungal activity against *T. mentagrophytes*, *T. rubrum*, *C. tropicalis*, *C. albicans* and *A. niger* as revealed by a large zone of inhibition (Table 1). This step resulted in protein recovery of approximately 32% (Table 2) with an increase in activity as indicated by a larger zone of inhibition compared with those generated by crude protein (Table 1). The DEAE-cellulose purified proteins in pooled fractions with antifungal activity were then further purified by gel filtration chromatography on a Sephadex G-25 column. The elution profile of proteins with total 7 peaks, designated as F1 to F7 were obtained (Figure 1b). Fractions were pooled and concentrated before continuing the assay. Sample from F4 showed major activity against 5 fungi and yeasts. The high activity was observed against *T. mentagrophytes*, *T. rubrum*, *C. tropicalis* and *C. albicans*, while the activity against *A. niger* is relatively low as indicated by smaller zone of inhibition. The zones of inhibition generated by sample from F4 against *T. mentagrophytes*, *T. rubrum*, *C. tropicalis*, *C. albicans* and *A. niger* were found to be significantly comparable to the zone of inhibition from Ketoconazole 20 µg/disc (Figure 2). Moreover, the activity was stronger compared with eluted fraction 0.5 M NaCl from DEAE-cellulose (Table 1). However, it has no antibacterial activity when tested against Gram positive

(*S. aureus* ATCC 6538 and *B. subtilis* ATCC 6633) as well as Gram negative (*E. coli* ATCC 35218, *K. pneumoniae* ATCC 4352 and *P. aeruginosa* ATCC 15442) bacteria (data not shown). The SDS-PAGE analysis of sample from F4 eluted from gel filtration revealed a single band with a molecular mass of approximately 17 kDa (Figure 3). The recovery of protein in this final step of purification was about 6% (Table 2).

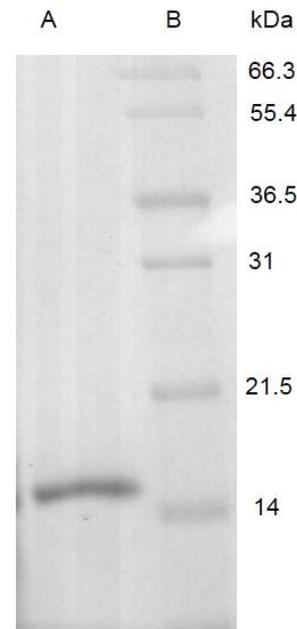


Figure 3: Determination of molecular mass by SDS-PAGE. The molecular mass of purified peptide from peak F4 (G25 column) shown in lane A (left) was approximately 17 kDa. It exhibited a single band at the same position that lay between the 14.-and 21.5 kDa protein marker bands in lane B (right).

Effect of various concentrations on antifungal activity

The effect of concentration of purified peptide on antifungal activity was determined using various amounts of purified peptide from F4 (G-25) including 3.75, 7.5, 15, 30, 40, 50 µg in 30 µL sample solution to impregnate the

paper discs for antifungal assay. The size of inhibition zone increased when the concentration of purified peptide in the paper disc increased (Figure 4). The results indicated that the activity of purified peptide against *T. mentagrophytes*, *T. rubrum*, *C. tropicalis*, *C. albicans* and *A. niger* was concentration dependent.

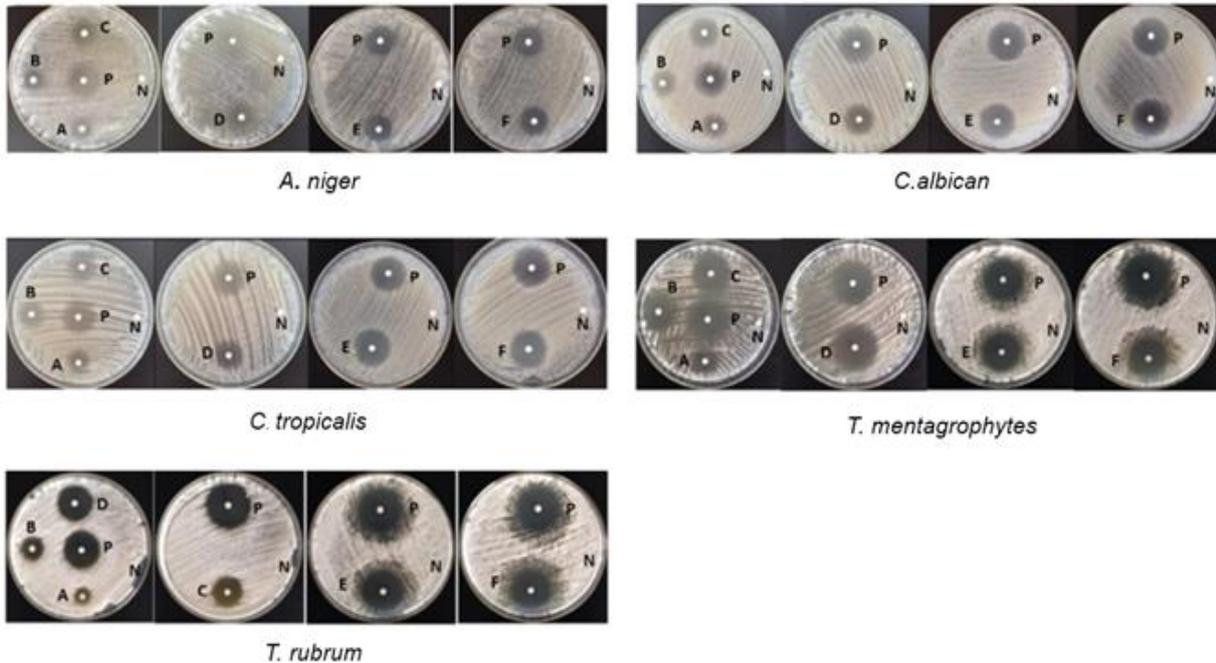


Figure 4: The zone of inhibition of purified peptide from peak F4 (G-25 column) with different protein concentration toward 5 different fungi. P, Ketoconazole was used as positive control (20 µg); N, 10 mM Tris-HCl buffer (pH 7.4) used as negative control and eluted sample peptide from F4 (G-25 column). Each sample was tested with various amount of purified peptide in 10 mM Tris-HCl buffer (pH 7.4). A, 3.75 µg; B, 7.5 µg; C, 15 µg; D, 30 µg; E, 40 µg and F, 50 µg of purified peptide.

DISCUSSION

Antifungal peptide has been isolated from fruiting bodies of the edible mushroom *L. squarrosulus* Mont. and purified by column chromatography on DEAE-cellulose and Sephadex G-25. Its chromatographic behavior on DEAE-cellulose is different from other mushroom antifungal proteins isolated earlier which are unadsorbed on the anion exchanger DEAE-cellulose (Wong *et al.*, 2010). This peptide is adsorbed on DEAE-cellulose and Sephadex G-25. It is noteworthy that essentially only two chromatographic steps are required to purify it to homogeneity. The molecular mass of mushroom antifungal proteins was reported in a wide range from 7 kDa in case of pleurostrin to 28 kDa in case of alveolarvin. (Wang and Ng, 2004; Chu *et al.*, 2005; Wong *et al.*, 2010). The purified peptide from mushroom *L. squarrosulus* Mont. displays a molecular mass of approximately 17 kDa as estimated by SDS-PAGE which is in the range of other mushroom antifungal proteins. Some antifungal proteins inhibit both fungi and bacteria, while others are lacking an antibacterial activity (Cammue

et al., 1995; Ngai and Ng, 2003). The antifungal peptide from mushroom *L. squarrosulus* Mont. also has no antibacterial activity against some test Gram positive and Gram negative bacteria used in this study. However, it has high activity against various species of fungal pathogen of human such as *T. mentagrophytes*, *T. rubrum*, *C. tropicalis* and *C. albicans*, while the activity against *A. niger* is low. Furthermore, the potency against fungal pathogen of human was found to be concentration dependent. It is interesting that among the high sensitive strains, *T. mentagrophytes*, *T. rubrum* and *C. tropicalis* are clinical isolates. Furthermore, its antifungal potency is significantly comparable to commercial antifungal drug, Ketoconazole.

In conclusion, the bioactive peptide from fruiting bodies of mushroom *L. squarrosulus* Mont. has been isolated and further purified to homogeneity with two simple chromatographic methods. This novel peptide has potent activity against various clinical isolates of fungal pathogen of human. It may be useful for pharmaceutical application.

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